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(54) Title: RNA RESPIRATORY SYNCYTIAL VIRUS VACCINES

(57) Abstract

A vector comprising a first DNA sequence which is complementary to at least part of an alphavirus RNA genome and having the complement of complete alphavirus DNA genome replication regions, a second DNA sequence encoding a paramyxovirus protein, particularly a respiratory syncytial virus fusion (RSV F) protein or an RSV F protein fragment that generates antibodies that specifically react with RSV F protein, the first and second DNA sequences being under the transcriptional control of a promoter is described. Such vector may be used to produce an RNA transcript which may be used to immunize a human host, to protect the host against disease caused by paramyxovirus, particularly respiratory syncytial virus, by administration to the host. The RNA transcript may be formed by linearization of the vector through cleavage at a unique restriction site in a plasmid vector and then transcribing the linear molecule.

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TITLE OF INVENTION
RNA RESPIRATORY SYNCYTIAL VIRUS VACCINES

FIELD OF INVENTION

5 The present invention relates to the field of paramyxoviridae vaccines and is particularly concerned with vaccines comprising RNA encoding the fusion (F) protein of respiratory syncytial virus (RSV).

BACKGROUND OF THE INVENTION

10 Human respiratory syncytial virus (RSV) has been identified as a major pathogen responsible for severe respiratory tract infections in infants, young children and the institutionalized elderly (refs. 1,2,3,4 -
15 throughout this application, various references are cited in parentheses to describe more fully the state of the art to which this invention pertains. Full bibliographic information for each citation is found at the end of the specification, immediately preceding the claims. The disclosures of these references are hereby
20 incorporated by reference into the present disclosure). Global mortality and morbidity figures indicate that there is an urgent need for an efficacious RSV vaccine (refs. 5,6). In the USA alone, approximately 100,000 children are hospitalized annually with severe cases of
25 pneumonia and bronchiolitis resulting from an RSV infection. Inpatient and ambulatory care for children with RSV infections has been estimated to cost in excess of \$340 million each year in the USA. The World Health Organization (WHO) and the National Institute of Allergy
30 and Infectious Disease (NIAID) vaccine advisory committees have ranked RSV second only to HIV for vaccine development. Both the annual morbidity and mortality figures as well as the staggering health care costs for managing RSV infections have provided the
35 incentive for aggressively pursuing the development of

efficacious RSV vaccines. However, such a vaccine is still not available.

Formalin-inactivated (FI-RSV) and live attenuated RSV vaccines have failed to demonstrate efficacy in clinical trials (refs. 7,8,9,10). Moreover, the formalin-inactivated RSV vaccine caused enhanced disease in some children following exposure to wild-type RSV (refs. 7,8,9,10). Elucidation of the mechanism(s) involved in the potentiation of RSV disease is important for the design of safe RSV vaccines, especially for the seronegative population. Recent experimental evidence suggests that an imbalance in cell-mediated responses may contribute to immunopotential. Enhanced histopathology observed in mice that were immunized with the FI-RSV and challenged with virus could be abrogated by depletion of CD4+ cells or both interleukin-4 (IL-4) and IL-10.

The RSV fusion (F) glycoprotein is one of the major immunogenic proteins of the virus. This envelope glycoprotein mediates both fusion of the virus to the host cell membrane and cell-to-cell spread of the virus (ref. 1). The F protein is synthesized as a precursor (F₀) molecule which is proteolytically cleaved to form a disulphide-linked dimer composed of the N-terminal F₂ and C-terminal F₁ moieties (ref. 11). The amino acid sequence of the F protein is highly conserved among RSV subgroups A and B and is a cross-protective antigen (refs. 6,12). In the baculovirus expression system, a truncated secreted version of the RSV F protein has been expressed in *Trichoplusia ni* insect cells (ref. 13). The recombinant protein was demonstrated to be protective in the cotton rats (ref. 13).

Studies on the development of live viral vaccines and glycoprotein subunit vaccines against parainfluenza virus infection are being pursued. Clinical trial results with a formalin-inactivated PIV types 1,2,3

vaccine demonstrated that this vaccine was not efficacious (refs. 14, 15, 16). Further development of chemically-inactivated vaccines was discontinued after clinical trials with a formalin-inactivated RSV vaccine demonstrated that not only was the vaccine not effective in preventing RSV infection but many of the vaccinees who later became infected with RSV suffered a more serious disease. Most of parainfluenza vaccine research has focussed on candidate PIV-3 vaccines (ref. 17) with significantly less work being reported for PIV-1 and PIV-2. Recent approaches to PIV-3 vaccines have included the use of the closely related bovine parainfluenza virus type 3 and the generation of attenuated viruses by cold-adaptation of the virus (refs. 18, 19, 20, 21).

Another approach to parainfluenza virus type 3 vaccine development is a subunit approach focusing on the surface glycoproteins hemagglutinin-neuraminidase (HN) and the fusion (F) protein (refs. 22, 23, 24). The HN antigen, a typical type II glycoprotein, exhibits both haemagglutination and neuraminidase activities and is responsible for the attachment of the virus to sialic acid containing host cell receptors. The type I F glycoprotein mediates fusion of the viral envelope with the cell membrane as well as cell to cell spread of the virus. It has recently been demonstrated that both the HN and F glycoproteins are required for membrane fusion. The F glycoprotein is synthesized as an inactive precursor (F) which is proteolytically cleaved into disulfide-linked F2 and F1 moieties. While the HN and F proteins of PIV-1, -2 and -3 are structurally similar, they are antigenically distinct. Neutralizing antibodies against the HN and F proteins of one of PIV type are not cross-protective. Thus, an effective PIV subunit vaccine must contain the HN and F glycoproteins from the three different types of parainfluenza viruses. Antibody to either glycoprotein is neutralizing in

vitro. A direct correlation has been observed between the level of neutralizing antibody titres and resistance to PIV-3 infections in infants. Native subunit vaccines for parainfluenza virus type 3 have investigated the protectiveness of the two surface glycoproteins. Typically, the glycoproteins are extracted from virus using non-ionic detergents and further purified using lectin affinity or immunoaffinity chromatographic methods. However, neither of these techniques may be entirely suitable for large scale production of vaccines under all circumstances. In small animal protection models (hamsters and cotton rats), immunization with the glycoproteins was demonstrated to prevent infection with live PIV-3 (refs. 25, 26, 27, 28, 29). The HN and F glycoproteins of PIV-3 have also been produced using recombinant DNA technology. HN and F glycoproteins have been produced in insect cells using the baculovirus expression system and by use of vaccinia virus and adenovirus recombinants (refs. 30, 31, 32, 33, 34). In the baculovirus expression system, both full-length and truncated forms of the PIV-3 glycoproteins as well as a chimeric F-HN fusion protein have been expressed. The recombinant proteins have been demonstrated to be protective in small animal models (see W091/00104, US Application No. 07/773,949 filed November 29, 1991, assigned to the assignee hereof).

Semliki Forest virus (SFV) is a member of the Alphavirus genus in the Togaviridae family. The mature virus particle contains a single copy of a ssRNA genome with a positive polarity that is 5'-capped and 3'-polyadenylated. It functions as an mRNA and naked RNA can start an infection when introduced into cells. Upon infection/transfection, the 5' two-thirds of the genome is translated into a polyprotein that is processed into the four nonstructural proteins (nsP1 to 4) by self cleavage. Once the ns proteins have been synthesized

they are responsible for replicating the plus-strand (42S) genome into full-length minus strands (ref. 35). These minus-strands then serve as templates for the synthesis of new plus-strand (42S) genomes and the 26S subgenomic mRNA (ref. 35). This subgenomic mRNA, which is colinear with the last one-third of the genome, encodes the SFV structural proteins. In 1991 Liljestrom and Garoff (ref. 36) designed a series of expression vectors based on the SFV cDNA replicon. These alphavirus vectors also are described in WO 92/10578, the disclosure of which is incorporated herein by reference. These vectors had the virus structural protein genes deleted to make the way for heterologous inserts, but preserved the nonstructural coding region for production of the nsP1 to 4 replicase complex. Short 5' and 3' sequence elements required for RNA replication were also preserved. A polylinker site was inserted downstream from the 26S promoter followed by translation stop sites in all three frames. An SpeI site was inserted just after the 3' end of the SFV cDNA for linearization of the plasmid for use in vitro transcription reactions.

Injections of SFV RNA encoding a heterologous protein have been shown to result in the expression of the foreign protein and the induction of antibody in a number of studies (refs. 37, 38). The use of SFV RNA inoculation to express foreign proteins for the purpose of immunization would have several of the advantages associated with plasmid DNA immunization. For example, SFV RNA encoding a viral antigen may be introduced in the presence of antibody to that virus without a loss in potency due to neutralization by antibodies to the virus. Also, because the protein is expressed in vivo the protein should have the same conformation as the protein expressed by the virus itself. Therefore, concerns about conformational changes which could occur

during protein purification leading to a loss in immunogenicity, protective epitopes and possibly immunopotential, could be avoided by nucleic acid immunization.

5 Immunization with SFV RNA also has several unique advantages over plasmid DNA immunization. SFV is one of the most efficiently replicating viruses known. After a few hours, up to 200,000 copies of the plus-RNAs can be made in a single cell. These SFV RNAs are so abundant
10 almost all of the cells ribosomes are enrolled in the synthesis of the SFV encoded proteins, thus overtaking host cell protein synthesis (ref. 36). Therefore, it should require a smaller dose of SFV RNA and less time to achieve a protective effect as compared to plasmid
15 DNA immunization. Secondly, RNA, unlike DNA, poses no potential threat of integrating into the cell genome. Thirdly, SFV RNA replication and expression occurs only in the cytoplasm of the cell. Therefore, problems involving nuclear transport and splicing associated with
20 nucleus-based expression systems (DNA immunization) are absent. Fourthly, since the replication of the SFV RNA is transient and RNA is quite labile, the SFV RNA will not persist for long periods after immunization like DNA plasmids.

25 In WO 95/27044, the disclosure of which is incorporated herein by reference, there is described the use of alphavirus cDNA vectors based on cDNA complementary to the alphavirus RNA sequence. Once transcribed from the cDNA under transcriptional control
30 of a heterologous promoter, the alphavirus RNA is able to self-replicate by means of its own replicase and thereby amplify the copy number of the transcribed recombinant RNA molecules.

35 In copending US Patent Application No. 08/476,397 filed June 7, 1995, (WO 96/40945), assigned to the assignee hereof and the disclosure of which is

incorporated herein by reference, there are described certain plasmid constructs used for DNA immunization which include forms of the RSV F gene. As seen therein, one plasmid pXL2 conferred complete protection on mice to challenge by live RSV when administered intranasally. This plasmid contains a gene encoding a truncated RSV F protein lacking the transmembrane portion of the protein, the immediate-early promoter enhancer and intron sequences of human cytomegalovirus (CMV) and the intron II sequences of rabbit β -globin to prevent aberrant splicing. The same plasmid construct but without the intron II sequences of rabbit β -globin, i.e. pXL1, provided only partial protection. Similarly, plasmid construct pXL4, which is the same as pXL2 except the RSV F gene encodes the full length RSV protein, provided partial protection while the corresponding construct lacking the intron II sequence of rabbit β -globin, i.e. pXL3, conferred no protection.

These data show that the absence of elements to reduce aberrant splicing adversely affects the protective ability of the plasmid. Aberrant splicing occurs during nuclear transcription of DNA to RNA. By employing RNA transcripts for immunization, the need for nuclear processing is avoided and aberrant splicing is unable to occur. This enables the use of the intron II sequences from non-human sources to be avoided.

The use of RNA transcripts for administration to the host enables there to be obtained total protection to challenge using a lower dose in less time than when employing the DNA plasmids described in USAN 08/476,397 (WO 96/40945). The use of the RNA transcripts avoids persistence of DNA in the immunized host and potential integration.

The ability to immunize against disease caused by RSV by immunization with naked SFV RNA encoding the RSV F protein, particularly the secreted version of the RSV

F protein, was unknown before the present invention and could not be predicted on the basis of the known prior art. Infection with RSV leads to serious disease. It would be useful and desirable to provide improved
5 vectors for *in vivo* administration of immunogenic preparations, including vaccines, for protection against disease caused by RSV. In particular, it would be desirable to provide vaccines that are immunogenic and protective in the elderly and paediatric human
10 populations, including seronegative infants, that do not cause disease enhancement (immunopotential).

SUMMARY OF THE INVENTION

The present invention provides novel immunogenic materials and immunization procedures based on such
15 novel materials for immunizing against disease caused by paramyxoviridae, including respiratory syncytial virus and parainfluenza virus. In particular, the present invention is directed towards the provision of RNA vaccines against disease caused by infection with
20 paramyxoviridae.

In accordance with one aspect of the present invention, there is provided a vector, comprising a first DNA sequence which is complementary to at least part of an alphavirus RNA genome and having the
25 complement of complete alphavirus RNA genome replication regions; a second DNA sequence encoding a paramyxovirus protein or a protein fragment that generates antibodies that specifically react with the paramyxovirus protein; the second DNA sequence being
30 inserted into a region of the first DNA sequence which is non-essential for replication; the first and second DNA sequences being under transcriptional control of a promoter.

The paramyxovirus protein may be selected from the
35 group consisting of a parainfluenza virus (PIV) and a respiratory syncytial virus (RSV). The PIV protein may

be PIV-1, PIV-2, PIV-3 or PIV-4, particularly the HN or F glycoproteins of PIV-3. The RSV protein particularly may be the F or G glycoprotein of RSV.

5 The second DNA sequence may encode a full length RSV F protein, or may encode a RSV F protein lacking the transmembrane anchor and cytoplasmic tail. The lack of the coding region for the transmembrane anchor and cytoplasmic tail results in a secreted form of the RSV F protein.

10 The second DNA sequence preferably encodes a RSV F protein and lacks a SpeI restriction site, and optionally, also lacking the transmembrane anchor and cytoplasmic tail encoding region. The absence of the SpeI restriction site may be carried out by mutating
15 nucleotide 194 (T) of the RSV F gene to a C, which eliminates the SpeI without altering the amino acid sequence. The nucleotide sequence (SEQ ID No: 1) and encoded amino acid sequence (SEQ ID No: 2) of the mutated truncated RSV F gene is shown in Figure 2.

20 The alphavirus preferably is a Semliki Forest virus and the first DNA sequence is the Semliki Forest viral sequence contained in plasmid pSFV1. The promoter used preferably is the SP6 promoter.

25 The vector may contain a unique restriction site permitting linearization of the vector without clearing the second nucleotide sequence and maintaining the first and second nucleotides sequences under transcriptional control of the promoter. The unique restriction site preferably is a SpeI site, particularly that derived
30 from pSFV1. The linearized form of the vector forms an embodiment herein.

The vector may be a plasmid vector, preferably one having the identifying characteristics of plasmid pMP37 (ATCC 97905) as shown in Figure 1C and, more preferably,
35 is the plasmid pMP37.

5 The mutant DNA sequence encoding an RSV F protein or a fragment thereof capable of inducing antibodies that specifically react with RSV F protein and lacking the SpeI restriction site present in the native DNA sequence, constitutes another aspect of the present invention. Such mutant DNA sequence lacking the SpeI site of the native sequence preferably is that shown in Figure 2 (SEQ ID No: 1) or one which encodes the amino acid sequence shown in Figure 2 (SEQ ID No: 2).

10 The novel vector provided herein may be linearized by cleavage at the unique restriction site and transcribed to an RNA transcript. In accordance with a further aspect of the invention, there is provided an RNA transcript of a vector as provided herein produced
15 by linearization and transcription.

 The RNA transcripts provided herein may be provided in the form of an immunogenic composition for *in vivo* administration to a host for the generation in the host of antibodies to paramyxovirus protein, such immunogenic
20 compositions comprising, as the active component thereof, an RNA transcript as provided herein. Such immunogenic compositions, which are provided in accordance with another aspect of the invention, may be formulated with any suitable pharmaceutically-acceptable
25 carrier for the *in vivo* administration and may produce a protective immune response.

 In a yet further aspect of the present invention, there is provided a method of immunizing a host against disease caused by infection with paramyxovirus, which
30 comprises administering to the host an effective amount of an RNA transcript as provided herein.

 The present invention also includes a novel method of using a gene encoding an RSV F protein or an fragment of an RSV F protein capable of generating antibodies
35 which specifically react with RSV F protein to protect a host against disease caused by infection with

respiratory syncytial virus, which comprises isolating said gene; operatively linking said gene to a DNA sequence which is complementary to at least part of an alphavirus RNA genome and having the complement of complete alphavirus RNA genome replication regions in a region of said DNA sequence which is non-essential for replication to form a plasmid vector wherein said gene and DNA sequence are under transcriptional control of a promoter; linearizing the plasmid vector while maintaining said gene and DNA sequence under said transcriptional control of the promoter; forming an RNA transcript of said linearized vector; and introducing said RNA transcript to said host.

Linearizing the plasmid vector is effected by clearing the plasmid vector at a unique restriction site therein at a location which permits the maintenance of the gene and the DNA sequence under the transcriptional control of the promoter. The unique restriction site may be an SpeI site, such as derived from plasmid pSFV1.

The plasmid vector employed preferably is plasmid pMP37 and the linearizing step is effected by cleavage at the SpeI site of plasmid pMP37 (see Figure 1C).

In addition, the present invention includes a method of producing a vaccine for protection of a host against disease caused by infection with respiratory syncytial virus (RSV), which comprises isolating a first DNA sequence encoding an RSV F protein from which the transmembrane anchor and cytoplasmic tail are absent and lacking any SpeI restriction site; operatively linking said first DNA sequence to a second DNA sequence which is complementary to at least part of an alphavirus RNA genome and having the complete alphavirus genome replication regions in a region of said second DNA sequence which is non-essential for replication to form a plasmid vector wherein said first

and second DNA sequences are under transcriptional control of a promoter; linearizing the plasmid vector while maintaining said first and second DNA sequences under said transcriptional control of the promoter; 5 forming a RNA transcript of said linearized vector; and formulating said RNA transcript as a vaccine for in vivo administration.

Linearizing the plasmid vector is effected by clearing the plasmid vector at a unique restriction 10 site therein at a location which permits the maintenance of the gene and the DNA sequence under the transcriptional control of the promoter. The unique restriction site may be an SpeI site, such as derived from plasmid pSFV1.

15 The plasmid vector employed preferably is plasmid pMP37 and the linearizing step is effected by cleavage at the SpeI site of plasmid pMP37.

Advantages of the present invention include the provision of RNA transcripts which are useful in 20 generating an immune response by in vivo administration.

BRIEF DESCRIPTION OF THE FIGURES

The present invention will be further understood from the following description with reference to the 25 drawings, in which:

Figures 1A, 1B and 1C show a scheme for construction of plasmid pMP37 used to generate the RSV-F RNA;

30 Figure 2 shows the nucleotide sequence (SEQ ID No: 1) and deduced amino acid sequence (SEQ ID No: 2) of a truncated RSV F gene lacking the transmembrane anchor and cytoplasmic tail and mutated at nucleotide 194 to eliminate the SpeI restriction site present in the unmutated gene;

35 Figure 3, comprising panels A, B and C, shows the anti-RSV F titres in sera from mice taken 4 weeks after

primary immunization and 2 weeks after boosting with the RSV F RNA. Panels A, B, and C show total IgG response, IgG1 response and IgG2a response respectively; and

Figure 4 shows the RSV-specific neutralizing antibody titres expressed as plaque reduction titres for various RSV preparations.

GENERAL DESCRIPTION OF INVENTION

As described above, the present invention, in general, relates to protection of hosts against disease caused by infection by paramyxovirus by RNA immunization using RNA transcripts formed from DNA vectors by linearization and transcription of the linearized vector. In particular, the invention is concerned with protection of hosts against disease caused by infection by respiratory syncytial virus (RSV), although not specifically limited thereto. The description which follows refers specifically to employing DNA sequences and RNA transcripts thereof encoding RSV F protein and fragments thereof which generate antibodies which specifically react with RSV F protein.

In this application, the term "RSV F protein" is used to define a full-length RSV F protein, including proteins having variations in their amino acid sequences including those naturally occurring in various strain of RSV and those introduced by PCR amplification of the encoding gene while retaining the immunogenic properties, a secreted form of the RSV F protein lacking a transmembrane anchor and cytoplasmic tail, as well as fragments of RSV F protein capable of generating antibodies which specifically react with RSV F protein and functional analogs of RSV F protein. In this application, a first protein is a "functional analog" of a second protein if the first protein is immunologically related to and/or has the same function as the second protein. The functional analog may be,

for example, a fragment of the protein or a substitution, addition or deletion mutant thereof.

5 A vector is constructed to contain a first DNA sequence which is complementary to at least part of an alphavirus RNA genome, specifically Semliki Forest virus, and having the complement of complete alphavirus RNA genome replication regions. A second DNA sequence encoding the RSV F protein is inserted into a region of the first DNA sequence which is non-essential for
10 replication. The first and second DNA sequences are under transcriptional control of a promoter. The resulting vector is linearized and an RNA transcript formed from the linearized vector.

The RNA transcripts provided herein, when
15 administered to an animal, including a human, replicate rapidly and effect *in vivo* RSV F protein expression, as demonstrated by an RVF-protein specific antibody response in the animal to which it is administered. Such antibodies may be employed, if desired, in the
20 detection of RSV protein in a sample.

As may be seen from the results detailed in the Examples below, the RNA transcripts provided a high anti-F IgG antibody titre with a IgG1/IgG2a ratio closely following the ratio obtained from immunization
25 with live virus. Immunization with the RNA transcripts protected the animals against live RSV challenge.

It is clearly apparent to one skilled in the art, that the various embodiments of the present invention have many applications in the fields of vaccination,
30 diagnosis and treatment of RSV infections. A further non-limiting discussion of such uses is further presented below.

1. Vaccine Preparation and Use

Immunogenic compositions, suitable to be used as
35 vaccines, may be prepared from the RSV F gene and vectors as disclosed herein. The vaccine elicits an

immune response in a subject which includes the production of anti-RSVF antibodies. Immunogenic compositions, including vaccines, containing the RNA transcripts may be prepared as injectables, in
5 physiologically-acceptable liquid solutions or emulsions for polynucleotide administration. The RNA transcripts associated with liposomes, such as lecithin liposomes or other liposomes known in the art, as a nucleic acid liposome (for example, as described in WO
10 93/24640, ref. 38) or the RNA may be associated with an adjuvant, as described in more detail below. Liposomes comprising cationic lipids interact spontaneously and rapidly with polyanions, such as DNA and RNA, resulting in liposome/nucleic acid complexes that capture up to
15 loo of the polynucleotide. In addition, the polycationic complexes fuse with cell membranes, resulting in an intracellular delivery of polynucleotide that bypasses the degradative enzymes of the lysosomal compartment. Published PCT application WO 94/27435
20 describes compositions for genetic immunization comprising cationic lipids and polynucleotides. Agents which assist in the cellular uptake of nucleic acid, such as calcium ions, viral proteins and other transfection facilitating agents, may advantageously be
25 used.

Polynucleotide immunogenic preparations may also be formulated as microcapsules, including biodegradable time-release particles. Thus, U.S. Patent 5,151,264 describes a particulate carrier of a
30 phospholipid/glycolipid/polysaccharide nature that has been termed Bio Vecteurs Supra Moléculaires (BVSM). The particulate carriers are intended to transport a variety of molecules having biological activity in one of the layers thereof.

35 U.S. Patent 5,075,109 describes encapsulation of the antigens trinitrophenylated keyhole limpet

hemocyanin and staphylococcal enterotoxin B in 50:50 poly (DL-lactidecoglycolide). Other polymers for encapsulation are suggested, such as poly(glycolide), poly(DL-lactide-coglycolide), copolyoxalates, 5 polycaprolactone, poly(lactide-co-caprolactone), poly(esteramides), polyorthoesters and poly(8-hydroxybutyric acid), and polyanhydrides.

Published PCT application WO 91/06282 describes a delivery vehicle comprising a plurality of bioadhesive 10 microspheres and antigens. The microspheres being of starch, gelatin, dextran, collagen or albumin. This delivery vehicle is particularly intended for the uptake of vaccine across the nasal mucosa. The delivery vehicle may additionally contain an absorption enhancer.

15 The RNA transcripts may be mixed with pharmaceutically acceptable excipients which are compatible therewith. Such excipients may include water, saline, dextrose, glycerol, ethanol, and combinations thereof. The immunogenic compositions and 20 vaccines may further contain auxiliary substances, such as wetting or emulsifying agents, pH buffering agents, or adjuvants to enhance the effectiveness thereof. Immunogenic compositions and vaccines may be administered parenterally, by injection subcutaneously, 25 intravenously, intradermally or intramuscularly, possibly following pretreatment of the injection site with a local anesthetic. Alternatively, the immunogenic compositions formed according to the present invention, may be formulated and delivered in a manner to evoke an 30 immune response at mucosal surfaces. Thus, the immunogenic composition may be administered to mucosal surfaces by, for example, the nasal or oral (intra-gastric) routes. Alternatively, other modes of administration including suppositories and oral 35 formulations may be desirable. For suppositories, binders and carriers may include, for example,

polyalkalene glycols or triglycerides. Oral formulations may include normally employed incipients, such as, for example, pharmaceutical grades of saccharine, cellulose and magnesium carbonate.

5 The immunogenic preparations and vaccines are administered in a manner compatible with the dosage formulation, and in such amount as will be therapeutically effective, protective and immunogenic. The quantity to be administered depends on the subject
10 to be treated, including, for example, the capacity of the individual's immune system to synthesize the RSV F protein and antibodies thereto, and if needed, to produce a cell-mediated immune response. Precise amounts of active ingredient required to be administered
15 depend on the judgment of the practitioner. However, suitable dosage ranges are readily determinable by one skilled in the art and may be of the order of about 1 µg to about 10 mg of the RSV F RNA. Suitable regimes for initial administration and booster doses are also
20 variable, but may include an initial administration followed by subsequent administrations. The dosage may also depend on the route of administration and will vary according to the size of the host. A vaccine which protects against only one pathogen is a monovalent
25 vaccine. Vaccines which contain antigenic material of several pathogens are combined vaccines and also belong to the present invention. Such combined vaccines contain, for example, material from various pathogens or from various strains of the same pathogen, or from
30 combinations of various pathogens.

Immunogenicity can be significantly improved if the vectors are co-administered with adjuvants, commonly used as 0.05 to 0.1 percent solution in phosphate-buffered saline. Adjuvants enhance the immunogenicity
35 of an antigen but are not necessarily immunogenic themselves. Adjuvants may act by retaining the antigen

locally near the site of administration to produce a depot effect facilitating a slow, sustained release of antigen to cells of the immune system. Adjuvants can also attract cells of the immune system to an antigen depot and stimulate such cells to elicit immune responses.

Immunostimulatory agents or adjuvants have been used for many years to improve the host immune responses to, for example, vaccines. Thus, adjuvants have been identified that enhance the immune response to antigens. Some of these adjuvants are toxic, however, and can cause undesirable side-effects, making them unsuitable for use in humans and many animals. Indeed, only aluminum hydroxide and aluminum phosphate (collectively commonly referred to as alum) are routinely used as adjuvants in human and veterinary vaccines.

A wide range of extrinsic adjuvants and other immunomodulating material can provoke potent immune responses to antigens. These include saponins complexed to membrane protein antigens to produce immune stimulating complexes (ISCOMS), plutonic polymers with mineral oil, killed mycobacteria in mineral oil, Freund's complete adjuvant, bacterial products, such as muramyl dipeptide (MDP) and lipopolysaccharide (LPS), as well as monophoryl lipid A, QS 21 and polyphosphazene.

In particular embodiments of the present invention, the RNA transcript comprising a first nucleotide sequence encoding an F protein of RSV may be delivered in conjunction with a targeting molecule to target the vector to selected cells including cells of the immune system.

The RNA transcript may be delivered to the host by a variety of procedures, for example, Tang et al. (ref. 39) disclosed that introduction of gold microprojectiles coated with DNA encoding bovine growth hormone (BGH) into the skin of mice resulted in production of anti-BGH

antibodies in the mice, while Furth et al. (ref. 40) showed that a jet injector could be used to transfect skin, muscle, fat and mammary tissues of living animals.

Biological Deposits

5 Certain vectors that contain the gene encoding RSV F protein and referred to herein have been deposited with the American Type Culture Collection (ATCC) located at 10801 University Boulevard, Manassas, Virginia 20110-2209, U.S.A., pursuant to the Budapest Treaty and prior
10 to the filing of this application.

Samples of the deposited plasmids will become available to the public upon grant of a patent based upon this United States patent application and all restrictions on access to the deposits will be removed
15 at that time. Non-viable deposits will be replaced in the event ATCC is unable to dispense the same. The invention described and claimed herein is not to be limited in scope by plasmids deposited, since the deposited embodiment is intended only as an illustration
20 of the invention. Any equivalent or similar plasmids that encode similar or equivalent antigens as described in this application are within the scope of this invention.

Deposit Summary

| | <u>Plasmid</u> | <u>ATCC Designation</u> | <u>Date Deposited</u> |
|----|----------------|-------------------------|-----------------------|
| 25 | pMP37 | 97905 | Feb. 27, 1997 |

EXAMPLES

The above disclosure generally describes the present invention. A more complete understanding can
30 be obtained by reference to the following specific Examples. These Examples are described solely for purposes of illustration and are not intended to limit the scope of the invention. Changes in form and substitution of equivalents are contemplated as
35 circumstances may suggest or render expedient. Although specific terms have been employed herein, such terms

are intended in a descriptive sense and not for purposes of limitations.

Methods of molecular genetics, protein biochemistry and immunology used but not explicitly described in this disclosure and these Examples are amply reported in the scientific literature and are well within the ability of those skilled in the art.

Example 1

This Example describes the construction of a Semliki Forest virus (SFV) expression vector containing a truncated version of the RSV F gene.

A truncated version of the RSV F gene was inserted into the SFV expression vector pSFV1 (Gibco BRL, Gaithersburg, MD, USA) according to the steps outlined in Figure 1. The RSV F gene was originally cloned from a subtype A RSV clinical isolate into plasmid pRSV F, as fully described in copending United States Patent Application No. 08/001,554 filed January 6, 1993, assigned to the assignee hereof and the disclosure of which is incorporated herein by reference, (ref. 41 and WO 93/14207). A fragment of the RSV F gene was excised from plasmid RSV F by digesting the plasmid with BspHI and EcoRI. The restriction enzyme BspHI cuts within the RSV F gene coding region, removing 48 amino acids from the C-terminus of the F protein. These amino acids make up most of the transmembrane domain and the entire cytoplasmic tail. The resulting 1.6 Kb truncated RSV F gene fragment was cloned into the EcoRI-BamHI sites of the Bluescript-based mammalian cell expression vector pMCR20 (Stratagene, La Jolla, CA) in a 3-way ligation with a linker, based upon the following sequence:

5' CATGACTTGATAATGAG 3' (SEQ ID No: 3)

3' TGAAC TATTACTCCTAG 5' (SEQ ID No: 4)

to generate plasmid pES13A, as described in the aforementioned USAN 08/001,554 (WO 93/14207). This linker adds a non-template encoded threonine to the

truncated RSV F protein C-terminus and inserts three successive stop codons at the end of the truncated gene.

5 The 1.6 Kb truncated RSV F gene fragment was then excised from plasmid pES13A by digesting with EcoRI and BamHI. In another 3-way ligation, the 1.6 Kb EcoRI-BamHI RSV F gene fragment was cloned into the BamHI site of the SFV expression vector pSFV1 with another linker, based upon the following sequence:

10 5' GATCCGCGCGCGCG 3' (SEQ ID No: 5)
 3' GCGCGCGCGCTTAA 5' (SEQ ID No: 6)

to generate plasmid pMP35. This plasmid contained two copies of the 1.6 Kb BamHI RSV F gene fragment. At this time, it was discovered that there was an SpeI site located in the RSV F gene fragment 193 bp from the upstream BamHI site. It is necessary to linearize a pSFV1 based plasmid with SpeI prior to its use in the in vitro transcription reaction described below. Therefore, the SpeI site in the RSV F gene needed to be removed and this was effected in the following manner.

20 The 1.6 Kb truncated RSV F gene fragment was excised from plasmid pMP35 by digesting with BamHI and ligated into the BamHI site of pUC19 to generate the plasmid pMP36. The Transformer™ site-directed mutagenesis kit (Clonetech, Palo Alto, CA, USA) and a primer, 5'- TGGTTGGTATACCAAGTGTATAACT (SEQ ID No: 7) were used, according to the manufacturer's instructions, to change nucleotide 194 from a T to a C. This change eliminates the SpeI site in the RSV F gene without affecting the amino acid sequence of the encoded RSV F protein. The sequence of plasmid pMP36A, which contains the altered RSV F gene, was determined by DNA sequence analysis. The 1.6 Kb truncated RSV F gene fragment was excised from plasmid pMP36A by digesting with BamHI and ligated into the BamHI site of pSFV1 to generate plasmid pMP37 (ATCC 97905). Proper orientation of the truncated RSV F gene was confirmed by restriction

mapping and DNA sequence analysis. Figure 2 shows the nucleotide sequence (SEQ ID No: 1) of the truncated RSV F gene BamHI fragment with the SpeI site eliminated and the amino acid sequence (SEQ ID No: 2) of the secreted RSV F protein encoded thereby.

Plasmid DNA was purified using plasmid DNA mide kits from Qiagen (Chatsworth, CA, USA), according to the manufacturer's instructions.

Example 2

This Example describes the preparation of SpeI linearized pMP37 required for the generation of SFV-RSVF RNA in in vitro transcription reactions and the preparation of SFV-RSVF RNA.

20 µg of plasmid pMP37, prepared as described in Example 1, was cut with SpeI in a 100 µL reaction containing 20 mM Tris-HCl (pH 7.4), 5 mM MgCl₂, 50 mM KCl and 30 units of SpeI (Gibco BRL, Gaithersburg, MD, USA).

SFV-RNA was generated from the linearized plasmid in a 300 µL in vitro transcription reaction using of the following materials :

40 mM Tris-HCl (pH 7.9)

6 mM MgCl₂

2 mM spermidine-(HCl)₃

1 mM DTT (dithiothreanol)

1 mM ATP (adenosine triphosphate)

1 mM GTP (guanosine triphosphate)

1 mM CTP (cytidine triphosphate)

1 mM UTP (uridine triphosphate)

1 mM m⁷G(5')ppp(5')G RNA cap analog (New England Biolabs, Mississauga, Ont., Canada)

360 units of RNasin® enzyme inhibitor (Promega, Madison, WI, USA)

270 units of SP6 RNA polymerase (Gibco BRL, Gaithersburg, MD, USA)

The reaction was incubated at 37°C for 50 minutes. The SFV-RSVF RNA so produced was purified from the salt, enzymes, unincorporated NTP's and cap analog by passing the reaction mix through CHROMA SPIN™-200 DEPC-H₂O columns (Clonetech, Palo Alto, CA, USA) (75 µL/column) according to the manufacturer's instructions. The purified RNA then was ethanol precipitated and resuspended in DEPC-treated H₂O to a final concentration of 1 µg/µL. The purified RNA was mixed with an equal volume of 2X PBS just prior to immunization.

Example 3

This Example describes the immunization of mice with SFV-RSVF RNA and the immunogenicity results obtained.

It has previously been shown that mice are susceptible to infection with RSV (ref. 42) and are a relevant animal model. The mice were immunized with the SFV-RSVF RNA prepared as described in Example 2, by the intramuscular (i.m.) route. The anterior tibialis muscles of five BALB/c mice (female 6 to 8 week old) (Jackson Lab., Bar Harbour, ME, USA) were bilaterally injected with 2 x 25 µg (0.5 µg/µL) of the PBS-directed SFV-RSVF RNA. Five days prior to RNA immunization, the muscles were treated with 2 x 50 µL of cardiotoxin (10 µM in PBS) (Latoxan, France). Treatment of muscles with cardiotoxin has previously been shown to enhance the uptake of DNA and enhance the immune response (ref. 43). These mice were boosted in an identical manner 4 weeks later (Table 1 below). The control groups were immunized with (1) SFV RNA expressing β-galactosidase (SFV-LacZ RNA), (2) SFV-RSVF RNA as prepared herein; (3) live RSV, (4) PBS with alum and RSV subunit preparation with alum. These mice were also boosted in an identical manner 4 weeks later (Table 1). The RSV subunit preparation, comprising RSV F, G and M proteins is described in

compending United States Patent Application No. 08/679,060 filed July 12, 1996, assigned to the assignee hereof and the disclosure of which is incorporated herein by reference (WO 98/02457).

5 Two weeks after the second immunization, mice were challenged intranasally with 10^6 plaque forming units (pfu) of the A2 strain of RSV (BG-4A). Animals were sacrificed 4 days later. Lungs were aseptically removed, weighed, and homogenized in 2 mL of complete
10 culture medium. The virus titre in lung homogenates was determined in duplicate using Vero cells, as previously described (ref. 44).

Sera was obtained from the mice at 4 and 6 weeks. Anti-RSV F antibody titres (IgG, IgG1 and IgG2a) in
15 these sera were determined by enzyme-linked immunosorbent assay (ELISA), as described in Example 4. The RSV-specific plaque reduction titres of these sera were determined as previously described (ref. 44).

The anti-RSV F antibody responses in the sera of
20 BALB/c mice that were immunized as outlined in Table 1 are summarized in Figure 3. The animals immunized with SFV-RSVF RNA, live RSV, or RSV subunit preparation + alum all had high total anti-RSV F IgG antibody titres in their serum at both 4 and 6 weeks (Figure 3, panel
25 A). However, the IgG1/IgG2a ratios differed markedly, as seen from Figure 3, panels B and C. The sera from animals that were immunized with live RSV had an anti-F IgG1/IgG2a ratio of approximately 0.69 after 6 weeks. This value is in contrast to the anti-RSV F IgG1/IgG2a
30 ratio obtained in mice after 6 weeks that were primed and boosted with the alum-adjuvanted RSV subunit preparation. In this case, the anti-RSV IgG1/IgG2a ratio was approximately 4.3. The anti-RSV F IgG1/IgG2a ratios obtained in mice immunized with SFV-RSVF RNA
35 after 6 weeks were 0.79. These results suggest that immunization of mice with the SFV-RSVF RNA results in

more of a Th-1 type response similar to that obtained with live RS virus rather than the Th-2 type response seen with the alum-adsorbed RSV subunit preparation.

As shown in Figure 4, the sera of mice that were primed and boosted with the various RSV preparations as outlined in Table 1, all had significant levels of RSV-specific neutralizing antibodies (groups 2, 3 and 5). In contrast to the placebo control animals (groups 1 and 4), the lower respiratory tract of mice that were immunized with SFV-RSVF RNA, live RSV, or the alum-adsorbed RSV subunit preparation, were completely protected against live RS virus challenge, as seen in Table 2.

Immunization of mice with the SFV-RSVF RNA protected mice against live RSV challenge. The protective ability of this SFV replicon was comparable to that induced by inoculation with live RSV or alum-adsorbed RSV subunits. The type of immune response generated appeared to be more of a Th-1 like response similar to that elicited by live RSV.

Example 4

This Example describes the determination of anti-RSV F antibody titres.

Nunc-MaxiSorp plate wells were coated overnight at room temperature with 2.5 ng of immunoaffinity-purified RSV F protein diluted in 0.05M carbonate-bicarbonate buffer, pH 9.6. Wells were blocked for non-specific binding by adding 0.1% BSA in PBS for 30 min. at room temperature, followed by two washes in a washing buffer of 0.1% BSA in PBS + 0.1% Tween 20. Serial two or four-fold dilutions of mouse serum was added to the wells. After a one hour incubation at room temperature, plates were washed five times with washing buffer, and horseradish peroxidase (HRP) labeled conjugate was added at the appropriate optimal dilution in washing buffer. The total IgG assay used F(ab')₂ goat antimouse IgG (H+L

specific)-HRP from Jackson Immuno Research Laboratory Inc. (Baltimore MD, USA). Sheep anti-mouse IgG1-HRP from Serotec (Toronto, Ontario, Canada) was used in the IgG1 assay and goat anti-mouse IgG2a from Caltag Laboratories (San Francisco, CA, USA) was used in the IgG2a assay. Following one hour incubation at room temperature, the plates were washed five times with washing buffer, and hydrogen peroxide (substrate) in the presence of tetramethylbenzidine was added. The reaction was stopped by adding 2 M sulfuric acid. The colour was read in a Multiscan Titertek plate reader at an optical density (OD) of 450 nm. The titre was taken as the reciprocal of the last dilution at which the OD was approximately double. This OD must be greater than the negative control of the assay at the starting dilution. The pre-immune serum of each animal was used as the negative control.

SUMMARY OF THE DISCLOSURE

In summary of this disclosure, there are provided novel vectors containing DNA sequences encoding a paramyxovirus protein, particularly a RSV F protein, which can be linearized and transcribed to RNA for in vivo administration to generate a protective immune response to disease caused by infection by paramyxovirus, particularly respiratory syncytial virus. Modifications are possible within the scope of this invention.

Table 1. Immunization protocol

| GROUP | PRIME | ROUTE OF INOCULATION | BOOST | ROUTE OF INOCULATION |
|-------|----------------------------------|----------------------|----------------------------------|----------------------|
| 1 | SFV-LacZ RNA ¹ | Intramuscular | SFV-LacZ RNA ¹ | Intramuscular |
| 2 | SFV-RSVF RNA ¹ | Intramuscular | SFV-RSVF RNA ¹ | Intramuscular |
| 3 | Live RSV ² | Intranasal | Live RSV ² | Intranasal |
| 4 | PBS + alum | Intramuscular | PBS + alum | Intramuscular |
| 5 | RSV subunits + alum ³ | Intramuscular | RSV subunits + alum ³ | Intramuscular |

Mice were inoculated with:

1. 25 µg of RNA was injected into each hind leg muscle in 50 µL of PBS
2. 2.5 X 10⁵ pfu of mouse-adapted A2 virus
3. 1 µg of RSV subunit vaccine adsorbed to alum (1.5 mg/dose)

Table 2

| GROUP | Antigen Formulation | | Mean Virus Lung Titre (log ₁₀ /g ± s.d.) | % Protection |
|-------|---------------------|---------------------|--------------------------------------------------------|--------------|
| | Prime | Boost | | |
| 1 | SFV-LacZ RNA | SFV-LacZ RNA | 4.18 ± 0.06 | 0 |
| 2 | SFV-RSVF RNA | SFV-RSVF RNA | ≤ 1.83 ± 0 | 100 |
| 3 | Live RSV | Live RSV | ≤ 1.83 ± 0 | 100 |
| 4 | PBS + alum | PBS + alum | 4.17 ± 0.17 | 0 |
| 5 | RSV subunits + alum | RSV subunits + alum | ≤ 1.83 ± 0 | 100 |

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CLAIMS

What we claim is:

1. A vector, comprising:
 - a first DNA sequence which is complementary to at least part of an alphavirus RNA genome and having the complement of complete alphavirus RNA genome replication regions,
 - a second DNA sequence encoding a paramyxovirus protein or a protein fragment that generates antibodies that specifically react with the paramyxovirus protein, said second DNA sequence being inserted into a region of said first DNA sequence which is non-essential for replication thereof,
 - said first and second DNA sequences being under transcriptional control of a promoter.
2. The vector of claim 1 wherein said paramyxovirus protein is selected from the group consisting of a parainfluenza virus (PIV) protein and a respiratory syncytial virus (RSV) protein.
3. The vector of claim 2 wherein said paramyxoviridae protein is a PIV protein selected from the group consisting of PIV-1, PIV-2, PIV-3 and PIV-4 protein.
4. The vector of claim 3 wherein the PIV protein is selected from the group consisting of the HN and F glycoproteins of PIV-3.
5. The vector of claim 2 wherein said paramyxoviridae protein is an RSV protein.
6. The vector of claim 5 wherein said RSV protein is selected from the group consisting of the F and G glycoproteins of RSV.
7. The vector of claim 1 wherein said second DNA sequence encodes a full-length RSV F protein.
8. The vector of claim 1 wherein said second DNA sequence encodes a RSV F protein lacking the transmembrane anchor and cytoplasmic tail.

9. The vector of claim 1 wherein said second DNA sequence encodes a RSV F protein and lacks a Spe I restriction site.
10. The vector of claim 1 wherein said second DNA sequence encodes a RSV F protein and lacks a Spe I restriction site and lacks a transmembrane anchor and cytoplasmic tail encoding region.
11. The vector of claim 10 wherein nucleotide 194 (T) of the RSV F gene is mutated to a C to eliminate the Spe I site in the RSV F gene.
12. The vector of claim 1 wherein said alphavirus is a Semliki Forest virus.
13. The vector of claim 12 wherein said first DNA sequence is the Semliki Forest virus sequence contained in plasmid pSFV1.
14. The vector of claim 12 wherein said promoter is the SP6 promoter.
15. The vector of claim 1 which is a plasmid vector having a unique restriction site permitting linearization of the vector without clearing the second DNA sequence.
16. The vector of claim 15 wherein the unique restriction site is SpeI site.
17. The vector of claim 16 wherein the SpeI site is derived from plasmid pSFV1.
18. The vector of claim 1 is in a linearized form.
19. The vector of claim 1 having the identifying characteristics of plasmid pMP37 (ATCC 97905) shown in Figure 1C.
20. The vector of claim 1 which is plasmid pMP37 (ATCC 97905).
21. A mutant DNA sequence encoding an RSV F protein or a fragment thereof capable of inducing antibodies that specifically react with RSV F protein, from which is about an SpeI restriction site present in the non-mutant sequence.

22. The mutant DNA sequence of claim 21 which lacks a transmembrane anchor and cytoplasmic tail encoding region.
23. The mutant of claim 21 wherein nucleotide 194 (T) of a non-mutant RSV F gene is mutated to a C to eliminate the SpeI site in the non-mutant RSV F gene.
24. A mutant DNA molecule sequence lacking an SpeI site present in the non-mutant sequence and encoding a truncated RSV F protein and having the DNA sequence shown in Figure 2 (SEQ ID No:1) or having a DNA sequence encoding the amino acid sequence shown in Figure 2 (SEQ ID No: 2).
25. An RNA transcript of the vector of claim 1.
26. The RNA transcript of claim 25 which is derived by linearization of a plasmid vector of claim 1 at a unique restriction site provided in the vector and permitting linearization of the vector without clearing the second DNA sequence and transcribing the linearized vector.
27. The RNA transcript of claim 26 wherein said unique restriction site is an SpeI site.
28. The RNA transcript of claim 25 wherein, in said vector, said second DNA sequence encodes a paramyxoviridae protein selected from the group consisting of a parainfluenza virus (PIV) protein and a respiratory syncytial virus (RSV) protein.
29. The RNA transcript of claim 28 wherein, in said vector, said second DNA sequence encodes a paramyxoviridae protein selected from the group consisting of PIV-1, PIV-2, PIV-3 and PIV-4.
30. The RNA transcript of claim 29 wherein, in said vector, said second DNA sequence encodes a paramyxoviridae protein selected from the group consisting of the HN and F glycoproteins of PIV-3.

31. The RNA transcript of claim 28 wherein, in said vector, said second DNA sequence encodes an RSV protein.
32. The RNA transcript of claim 31 wherein, in said vector, RSV protein is selected from the group consisting of the F and G glycoprotein of RSV.
33. The RNA transcript of claim 25 wherein, in said vector, said second DNA sequence encodes a full-length RSV F protein.
34. The RNA transcript of claim 25 wherein, in said vector, said second DNA sequence encodes a RSV F protein lacking the transmembrane anchor and cytoplasmic tail.
35. The RNA transcript of claim 25 wherein, in said vector, said second DNA sequence encodes a RSV F protein and lacks a Spe I restriction site.
36. The RNA transcript of claim 35 wherein, in said vector, said second DNA sequence encodes a RSV F protein and lacks a Spe I restriction site and lacks a transmembrane anchor and cytoplasmic tail encoding region.
37. The RNA transcript of claim 36 wherein, in said vector, nucleotide 194 (T) of the RSV F gene is mutated to a C to eliminate the Spe I site in the native RSV F gene.
38. The RNA transcript of claim 25 wherein, in said vector, said alphavirus is a Semliki Forest virus.
39. The RNA transcript of claim 38 wherein, in said vector, said first DNA sequence is the Semliki Forest virus sequence contained in plasmid pSFV1.
40. The RNA transcript of claim 38 wherein, in said vector, said promoter is the SP6 promoter.
41. The RNA transcript of claim 25, wherein, said vector has the identifying characteristics of plasmid pMP37 (ATCC 97905) shown in Figure 1C.

42. The RNA transcript of claim 25, wherein said vector is plasmid pMP37 (ATCC 97905).

43. An immunogenic composition for in vivo administration to a host for the generation in the host of antibodies to paramyxoviridae protein, comprising, as the active component thereof, an RNA transcript as claimed in claim 25.

44. The immunogenic composition of claim 43 for the generation of protective antibodies in the host.

45. A method of immunizing a host against disease caused by infection with paramyxovirus, which comprises administering to said host an effective amount of an RNA transcript as claimed in claim 25.

46. A method of using a gene encoding an RSV F protein, which comprises:

isolating said gene,

operatively linking said gene to a DNA sequence which is complementary to at least part of an alphavirus RNA genome and having the complement of complete alphavirus RNA genome replication regions in a region of said DNA sequence which is non-essential for replication to form a plasmid vector wherein said gene and DNA sequence are under transcriptional control of a promoter,

linearizing the plasmid vector while maintaining said gene and DNA sequence under said transcriptional control of the promoter,

forming an RNA transcript of said linearized vector, and

introducing said RNA transcript to said host.

47. The method of claim 46 wherein said linearizing of the plasmid vector is effected by clearing the plasmid vector at a unique restriction site therein at a location which permits the maintenance of the gene and DNA sequence under transcriptional control of the promoter.

48. The method of claim 47 wherein the unique restriction site is an SpeI site.

49. The method of claim 46 wherein said vector is plasmid pMP37 (ATCC 97905) and is linearized by cleavage at the SpeI site therein.

50. A method of producing a vaccine for protection of a host against disease caused by infection with respiratory syncytial virus (RSV), which comprises:

isolating a first DNA sequence encoding an RSV F protein from which the transmembrane anchor and cytoplasmic tail are absent and lacking any SpeI restriction site,

operatively linking said first DNA sequence to a second DNA sequence which is complementary to at least part of an alphavirus RNA genome and having the complete alphavirus genome replication regions in a region of said second DNA sequence which is non-essential for replication to form a plasmid vector wherein said first and second DNA sequences are under transcriptional control of a promoter,

linearizing the plasmid vector while maintaining said first and second DNA sequences under said transcriptional control of the promoter,

forming a RNA transcript of said linearized vector, and

formulating said RNA transcript as a vaccine for *in vivo* administration.

51. The method of claim 50 wherein said linearizing of the plasmid vector is effected by clearing the plasmid vector at a unique restriction site therein at a location which permits the maintenance of the gene and DNA sequence under transcriptional control of the promoter.

52. The method of claim 51 wherein the unique restriction site is an SpeI site.

53. The method of claim 50 wherein said vector is plasmid pMP37 (ATCC 97905) and is linearized by cleavage at the SpeI site.

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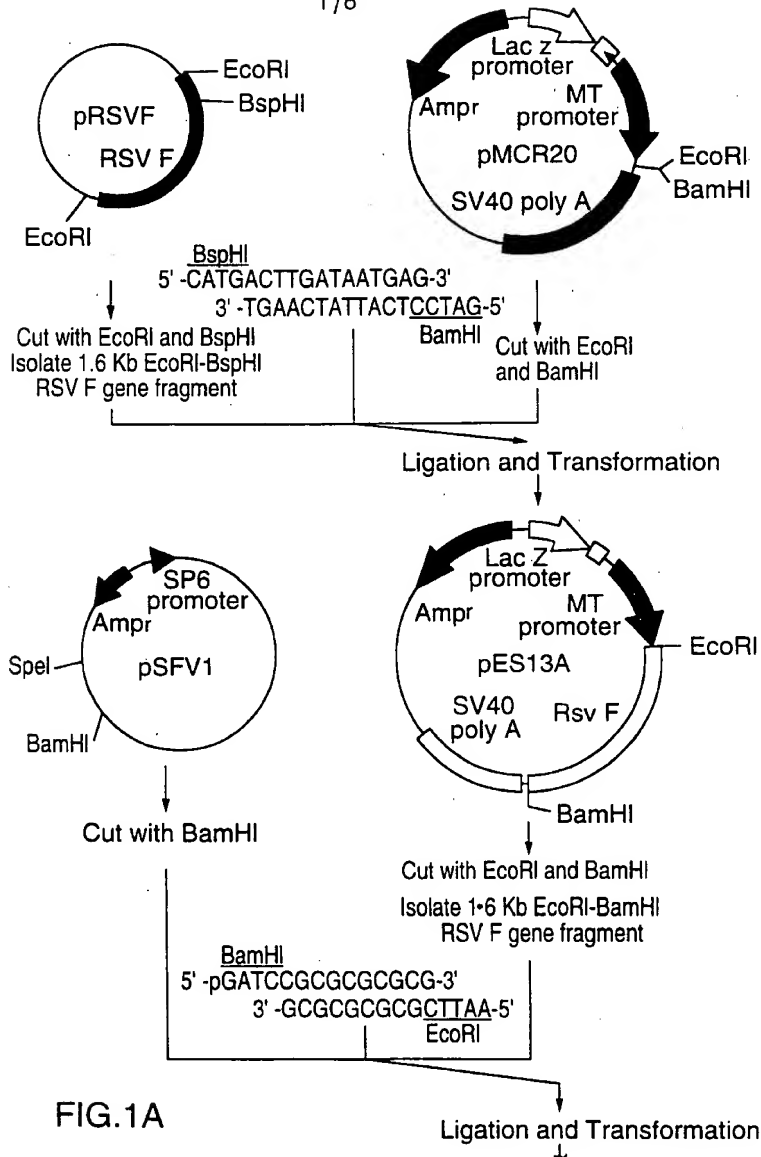


FIG.1A

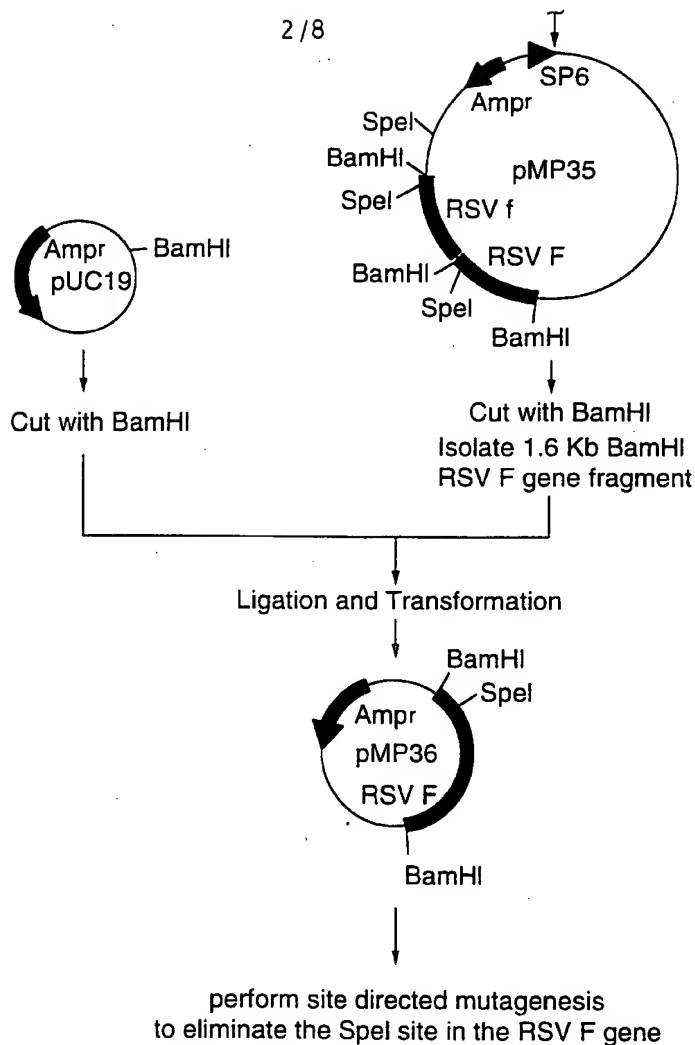


FIG.1B

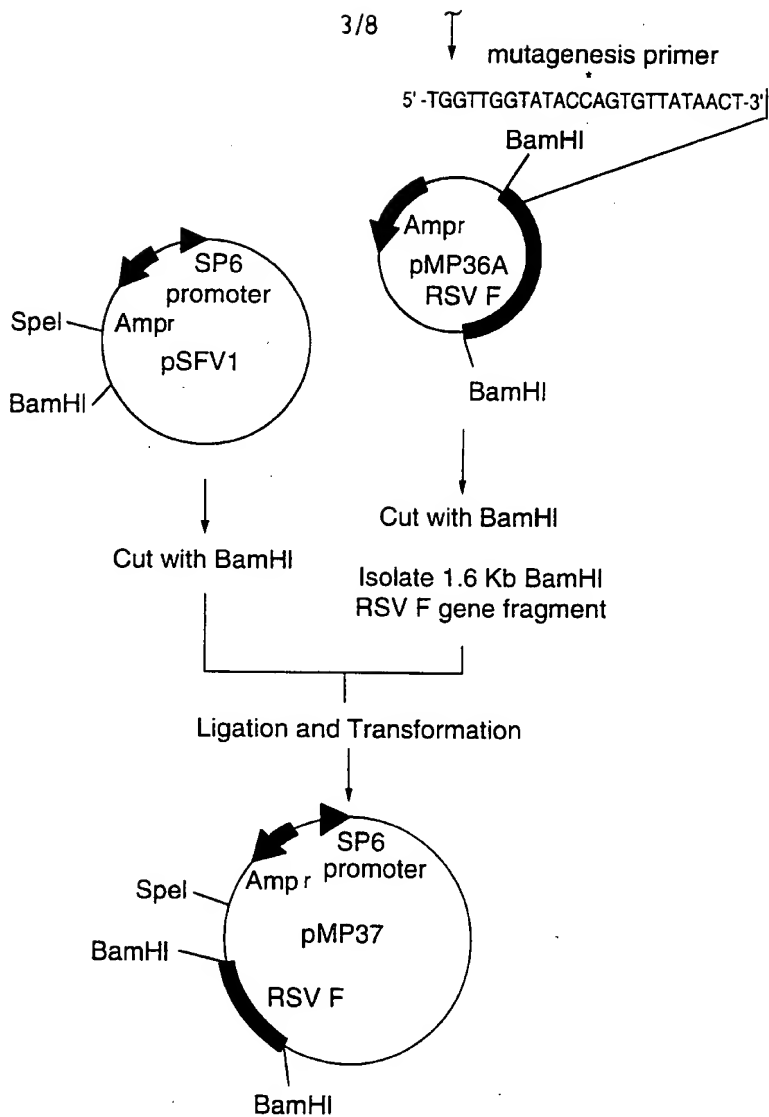


FIG.1C

FIG.2A

0 GATCCGGCGGCGGAATTCGGCACGAGTAACAATGGAGTTGCTAATCCTCAAAGCAAATG 60
 0
 61 CAATTACCACAAATCCTCACTGCAGTCACATTTTGTGTTTCTGTTCAAAAACATCACTG 120
 10
 121 AAGAAATTTTATCAATCAACATGCAGTGCAGTTAGCAAAAGGCTATCTTTAGTGTCTCTGAGAA 180
 30
 181 CTGGTTGGTATACCAAGTGTATATACTATAGAAATTAAGTAATATCAAGGAAAAATAAGTGTA 240
 50
 241 ATGGAACAGATGCTAAGGTAAAAATTGATAAAACAAGAAATTAGATAAAATATAAAAAATGCTG 300
 70
 301 TAACAGAATTGCAGTTGCTCATGCAAAAGCACACACCACCAACAAACAATCGAGCCAGAAGAG 360
 90
 481 AACTACCAAGGTTTATGAATTATACACTCAACAATGCCCCAAAAAACCAATGTAAACATTAA 420
 110
 421 GCAAGAAAAGGAAAAGAAGATTCTTGGTTTTTTTGTAGGTGTTGGATCTGCAATCGCCA 480
 130
 481 GTGGCGTTGCTGTATCTAAGGTCTGCACCTAGAGGGGAAGTGAACAAGATCAAAAAGTG 540
 150

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FIG.2B

541 CTCTACTATCCACAAACAAGGCTGTAGTCAGCTTATCAAATGGAGTTAGTGTCTTAACCA 600
 170
 601 GCAAAAGTGTAGACCTCAAAAACATATATAGATAAAACAATTGTACCTATTGTGAACAAGC 660
 190
 661 AAAGCTGCAGCATATCAAATATAGAAACGTGTATAGAGTTCCAAACAAAAGAACACAGAC 720
 210
 721 TACTAGAGATTACCAGGGAATTTAGTGTTAATGCAGGTGTAACCTACACCTGTAAGCACTT 780
 230
 781 ACATGTTAACTAATAGTGAATTTATGTTCATTAATCAATGATATGCCCTATAACAAAATGATC 840
 250
 841 AGAAAAAGTTAATGTCCAAACAATGTTCAAATAGTTAGACAGCAAAAGTTACTCTATCATGT 900
 270
 901 CCATAATAAAGAGGAAGTCTTAGCATATGTAGTACAATTACCACCTATATGGTGTATAG 960
 290
 961 ATACACCCTGTTGGAAACTACACACATCCCCCTCTATGTACAACCAACACAAAAGAAGGGT 1020
 310 D T P C W K L H T S P L C T T N T K E G 329
 1021 CCAACATCTGTTTAAACAAGAAGTACACAGAGGATGGTACTGTGACAATGCAGGATCAGTAT 1080
 330 S N I C L T R T D R G W Y C D N A G S V 349

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FIG.2C

1081 CTTTCCTCCACAAGCTGAAAACATGTAAAGTTCAATCAAATCGAGTATTTTGTGACACAA 1140
 350 S F F P Q A E T C K V Q S N R V F C D T 369
 1141 TGAACAGTTTAAACATTACCAAGTGAATAAATCTCTGCAATGTTGACATATTTCAACCCCA 1200
 370 M N S L T L P S E I N L C N V D I F N P 389
 1201 AATATGATTGTAAATATGACTTCAAAAAACAGATGTAAGCAGCTCCGTTATCACATCTC 1260
 390 K Y D C K I M T S K T D V S S S V I T S 409
 1261 TAGGAGCCATTGTGTCATGCTATGGCAAAACTAAATGTACAGCATCCAATAAAAAATCGTG 1320
 410 L G A I V S C Y G K T K C T A S N K N R 429
 1321 GAATCATAAAGACATTTTCTAACGGGTGCGATTATGTATCAAAATAAAGGGATGGACACTG 1380
 430 G I I K T F S N G C D Y V S N K G M D T 449
 1381 TGTCTGTAGGTAACACATTATATATGTAAATAAGCAAGGTAAGTCTCTATGTAA 1440
 450 V S V G N T L Y Y V N K Q E G K S L Y V 469
 1441 AAGGTGAACCAATAATAATTTCTATGACCCATTAGTATTTCCCTCTGATGAATTTGATG 1500
 470 K G E P I I N F Y D P L V F P S D E F D 489
 1501 CATCAATATCTCAAGTCAACGAGAAGATTAAACCAGAGCCTAGCATTTTATTCGTAATCCG 1560
 490 A S I S Q V N E K I N Q S L A F I R K S 509
 1561 ATGAATTATTACATAATGTAAATGCTGGTAAATCCACCACAAATATCATGACTTGATAAT 1620
 510 D E L L H N V N A G K S T T N I M T * 527
 1621 GAG 1623

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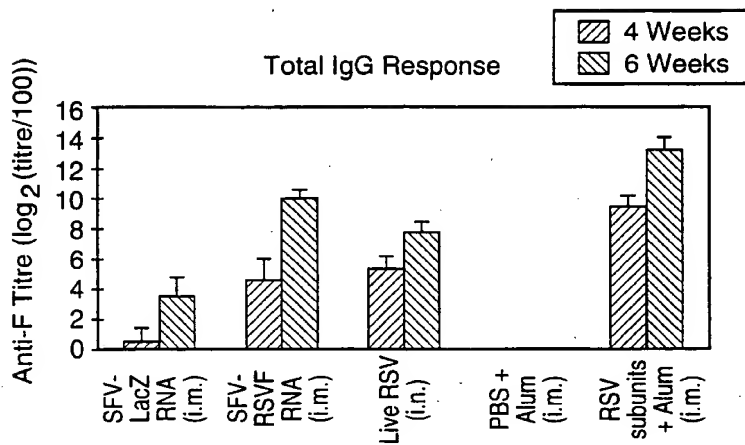


FIG.3A

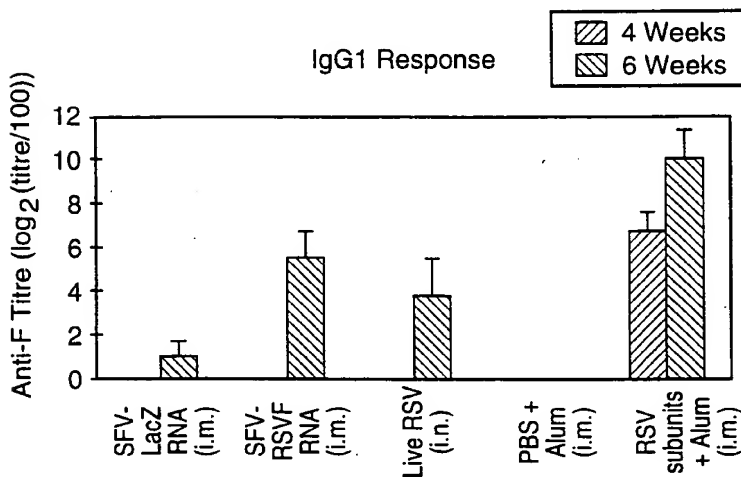


FIG.3B

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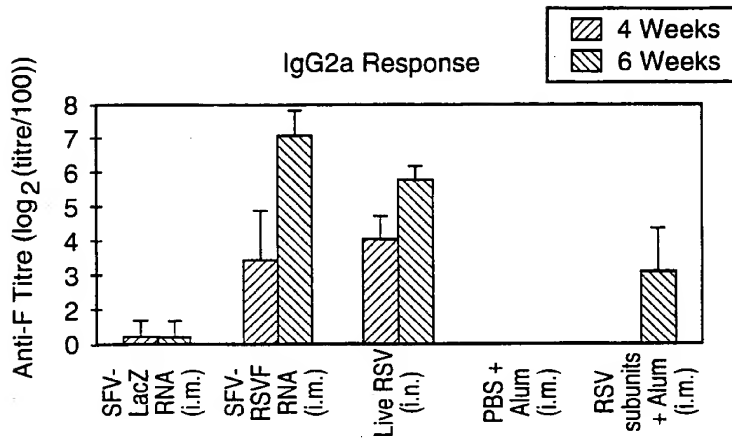


FIG.3C

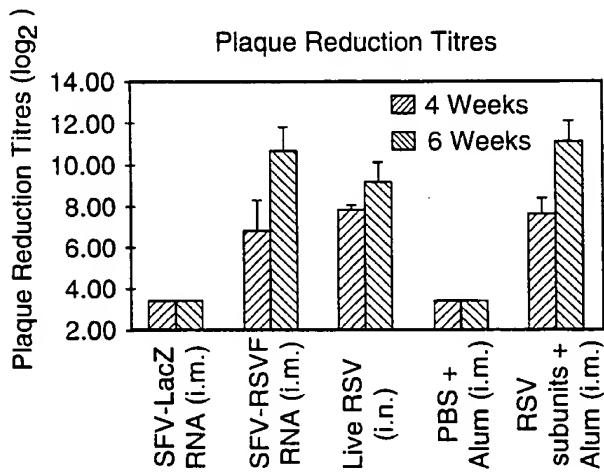


FIG.4

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

(A) NAME: CONNAUGHT LABORATORIES LIMITED
(B) STREET: 1755 Steeles Avenue West
(C) CITY: North York
(D) STATE: Ontario
(E) COUNTRY: Canada
(F) POSTAL CODE (ZIP): M2R 3T4

(A) NAME: PARRINGTON, Mark
(B) STREET: 45 Martin Street
(C) CITY: Bradford
(D) STATE: Ontario
(E) COUNTRY: Canada
(F) POSTAL CODE (ZIP): L3Z 1Z4

(ii) TITLE OF INVENTION: RNA RESPIRATORY SYNCYTIAL VIRUS VACCINES

(iii) NUMBER OF SEQUENCES: 7

(iv) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1623 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

| | | | | | | |
|------------|------------|-------------|------------|------------|------------|-----|
| GATCCGCGCG | CGCGAATTCG | GCACGAGTAA | CAATGGAGTT | GCTAATCCTC | AAAGCAAATG | 60 |
| CAATTACCAC | AATCCTCACT | GCACTCACAT | TTTGTTTTGC | TTCTGGTCAA | AACATCACTG | 120 |
| AAGAATTTTA | TCAATCAACA | TGCAGTGCAG | TTAGCAAAGG | CTATCTTAGT | GCTCTGAGAA | 180 |
| CTGGTTGGTA | TACCAAGTGT | ATAACTATAG | AATTAAGTAA | TATCAAGGAA | AATAAGTGTA | 240 |
| ATGGAACAGA | TGCTAAGGTA | AAATTGATAA | AACAAGAATT | AGATAAATAT | AAAAATGCTG | 300 |
| TAACAGAATT | GCAGTTGCTC | ATGCAAAGCA | CACCACCAAC | AAACAATCGA | GCCAGAAGAG | 360 |
| AACTACCAAG | GTTTATGAAT | TATACTACTCA | ACAATGCCAA | AAAAACCAAT | GTAACATTAA | 420 |
| GCAAGAAAAG | GAAAAGAAGA | TTTCTGGTT | TTTGTTAGG | TGTTGGATCT | GCAATCGCCA | 480 |
| GTGGCGTTGC | TGTATCTAAG | GTCTGCACC | TAGAAGGGA | AGTGAACAG | ATCAAAAGTG | 540 |
| CTCTACTATC | CACAAACAAG | GCTGTAGTCA | GCTTATCAAA | TGGAGTTAGT | GTCTTAACCA | 600 |

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GCAAAGTGTT AGACCTCAAA AACTATATAG ATAAACAATT GTTACCTATT GTGAACAAGC      660
AAGGTGCAG CATATCAAA ATAGAACTG TGATAGAGTT CCAACAAAG AACACAGAC      720
TACTAGAGAT TACCAGGAA TTTAGTGTTA ATGCAGGTGT AACTACACCT GTAAGCACTT      780
ACATGTTAAC TAATAGTGAA TTATTGTCAT TAATCAATGA TATGCCTATA ACAAAATGATC      840
AGAAAAAGTT AATGTCCAAC AATGTTCAAA TAGTTAGACA GCAAAGTTAC TCTATCATGT      900
CCATAATAAA AGAGGAAGTC TTAGCATATG TAGTACAATT ACCACTATAT GGTGTTATAG      960
ATACACCCCTG TTGAAACTA CACACATCCC CTCTATGTAC AACCAACACA AAAGAAGGGT    1020
CCAACATCTG TTTAACAAGA ACTGACAGAG GATGGTACTG TGACAATGCA GGATCAGTAT    1080
CTTTCTTCCC ACAAGCTGAA ACATGTAAAG TTCAATCAAA TCGAGTATTT TGTGACACAA    1140
TGAACAGTTT AACATTACCA AGTGAATAA ATCTCTGCAA TGTGACATA TTCAACCCCA    1200
AATATGATTG TAAAATTATG ACTTCAAAAA CAGATGTAAG CAGCTCCGTT ATCACATCTC    1260
TAGGAGCCAT TGTGTCATCG TATGGCAAAA CTAAATGTAC AGCATCCAAT AAAAATCGTG    1320
GAATCATAAA GACATTTTCT AACGGGTGCG ATTATGTATC AAATAAAGGG ATGGACACTG    1380
TGCTGTAGG TAACACATT AATTATGTAA ATAAGCAAGA AGGTAAAAGT CTCTATGTAA    1440
AAGGTGAACC AATAATAAAT TTCTATGACC CATTAGTATT CCCCTCTGAT GAATTTGATG    1500
CATCAATATC TCAAGTCAAC GAGAAGATTA ACCAGAGCCT AGCATTTTAT CGTAAATCCG    1560
ATGAATTATT ACATAATGTA AATGCTGGTA ARTCCACCAC AAATATCATG ACTTGATAAT    1620
GAG                                                                    1623

```

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 527 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```

Met Glu Leu Leu Ile Leu Lys Ala Asn Ala Ile Thr Thr Ile Leu Thr
 1           5           10           15
Ala Val Thr Phe Cys Phe Ala Ser Gly Gln Asn Ile Thr Glu Glu Phe
          20           25           30
Tyr Gln Ser Thr Cys Ser Ala Val Ser Lys Gly Tyr Leu Ser Ala Leu
          35           40           45
Arg Thr Gly Trp Tyr Thr Ser Val Ile Thr Ile Glu Leu Ser Asn Ile
          50           55           60
Lys Glu Asn Lys Cys Asn Gly Thr Asp Ala Lys Val Lys Leu Ile Lys
65           70           75           80

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Gln Glu Leu Asp Lys Tyr Lys Asn Ala Val Thr Glu Leu Gln Leu Leu
 85 90 95
 Met Gln Ser Thr Pro Pro Thr Asn Asn Arg Ala Arg Arg Glu Leu Pro
 100 105 110
 Arg Phe Met Asn Tyr Thr Leu Asn Asn Ala Lys Lys Thr Asn Val Thr
 115 120 125
 Leu Ser Lys Lys Arg Lys Arg Arg Phe Leu Gly Phe Leu Leu Gly Val
 130 135 140
 Gly Ser Ala Ile Ala Ser Gly Val Ala Val Ser Lys Val Leu His Leu
 145 150 155 160
 Glu Gly Glu Val Asn Lys Ile Lys Ser Ala Leu Leu Ser Thr Asn Lys
 165 170 175
 Ala Val Val Ser Leu Ser Asn Gly Val Ser Val Leu Thr Ser Lys Val
 180 185 190
 Leu Asp Leu Lys Asn Tyr Ile Asp Lys Gln Leu Leu Pro Ile Val Asn
 195 200 205
 Lys Gln Ser Cys Ser Ile Ser Asn Ile Glu Thr Val Ile Glu Phe Gln
 210 215 220
 Gln Lys Asn Asn Arg Leu Leu Glu Ile Thr Arg Glu Phe Ser Val Asn
 225 230 235 240
 Ala Gly Val Thr Thr Pro Val Ser Thr Tyr Met Leu Thr Asn Ser Glu
 245 250 255
 Leu Leu Ser Leu Ile Asn Asp Met Pro Ile Thr Asn Asp Gln Lys Lys
 260 265 270
 Leu Met Ser Asn Asn Val Gln Ile Val Arg Gln Gln Ser Tyr Ser Ile
 275 280 285
 Met Ser Ile Ile Lys Glu Glu Val Leu Ala Tyr Val Val Gln Leu Pro
 290 295 300
 Leu Tyr Gly Val Ile Asp Thr Pro Cys Trp Lys Leu His Thr Ser Pro
 305 310 315 320
 Leu Cys Thr Thr Asn Thr Lys Glu Gly Ser Asn Ile Cys Leu Thr Arg
 325 330 335
 Thr Asp Arg Gly Trp Tyr Cys Asp Asn Ala Gly Ser Val Ser Phe Phe
 340 345 350
 Pro Gln Ala Glu Thr Cys Lys Val Gln Ser Asn Arg Val Phe Cys Asp
 355 360 365
 Thr Met Asn Ser Leu Thr Leu Pro Ser Glu Ile Asn Leu Cys Asn Val
 370 375 380
 Asp Ile Phe Asn Pro Lys Tyr Asp Cys Lys Ile Met Thr Ser Lys Thr
 385 390 395 400
 Asp Val Ser Ser Ser Val Ile Thr Ser Leu Gly Ala Ile Val Ser Cys
 405 410 415

4

Tyr Gly Lys Thr Lys Cys Thr Ala Ser Asn Lys Asn Arg Gly Ile Ile
 420 425 430
 Lys Thr Phe Ser Asn Gly Cys Asp Tyr Val Ser Asn Lys Gly Met Asp
 435 440 445
 Thr Val Ser Val Gly Asn Thr Leu Tyr Tyr Val Asn Lys Gln Glu Gly
 450 455 460
 Lys Ser Leu Tyr Val Lys Gly Glu Pro Ile Ile Asn Phe Tyr Asp Pro
 465 470 475 480
 Leu Val Phe Pro Ser Asp Glu Phe Asp Ala Ser Ile Ser Gln Val Asn
 485 490 495
 Glu Lys Ile Asn Gln Ser Leu Ala Phe Ile Arg Lys Ser Asp Glu Leu
 500 505 510
 Leu His Asn Val Asn Ala Gly Lys Ser Thr Thr Asn Ile Met Thr
 515 520 525

(2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 17 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CATGACTTGA TAATGAG

17

(2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 17 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

TGAACATATTA CTCCTAG

17

(2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 14 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GATCCGCGCG CGCG

14

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 14 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GCGCGCGCGC TTAA

14

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 25 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

TGGTTGGTAT ACCAGTGTTA TAACT

25

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/CA 98/00840

| A. CLASSIFICATION OF SUBJECT MATTER IPC 6 C12N15/86 C12N15/45 C07K14/115 C07K14/135 A61K31/70 | | |
|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------|
| According to International Patent Classification (IPC) or to both national classification and IPC | | |
| B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) IPC 6 C12N C07K A61K | | |
| Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched | | |
| Electronic data base consulted during the international search (name of data base and, where practical, search terms used) | | |
| C. DOCUMENTS CONSIDERED TO BE RELEVANT | | |
| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
| Y | WO 96 40945 A (CONNAUGHT LAB ;LI XIAOMAO (CA); EWASYSHYN MARY E (CA); SAMBHARA SU) 19 December 1996 cited in the application see the whole document | 1-53 |
| Y | LILJESTROEM P. ET AL.: "A NEW GENERATION OF ANIMAL CELL EXPRESSION VECTORS BASED ON THE SEMLIKI FOREST VIRUS REPLICON" BIO/TECHNOLOGY, vol. 9, December 1991, pages 1356-1361, XP000616021 cited in the application see the whole document | 1-53 |
| A | US 5 614 372 A (LILJA HANS ET AL) 25 March 1997 see column 5, line 61 - line 65 | 1-53 |
| -/-- | | |
| <input checked="" type="checkbox"/> Further documents are listed in the continuation of box C. | | <input checked="" type="checkbox"/> Patent family members are listed in annex. |
| * Special categories of cited documents : "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "Z" document member of the same patent family | | |
| Date of the actual completion of the international search 8 January 1999 | | Date of mailing of the international search report 22/01/1999 |
| Name and mailing address of the ISA European Patent Office, P.B. 5618 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 851 epo nl, Fax: (+31-70) 340-3016 | | Authorized officer Mandl, B |

INTERNATIONAL SEARCH REPORT

International Application No
PCT/CA 98/00840

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------|
| A | SCHLESINGER S.: "ALPHAVIRUSES - VECTORS FOR THE EXPRESSION OF HETEROLOGOUS GENES" TRENDS IN BIOTECHNOLOGY, vol. 11, no. 1, 1 January 1993, pages 18-22, XP000358625 see page 20, left-hand column, last paragraph - right-hand column, paragraph 1 ----- | 1-53 |
| A | WO 96 17072 A (VIAGENE INC) 6 June 1996 see the whole document, especially page 38, lines 20-31 and page 61 lines 12-32 ----- | 1-53 |
| A | ZHOU X. ET AL.: "Self-replicating Semliki-Forest virus RNA as recombinant vaccine." VACCINE, vol. 12, no. 16, 1994, pages 1510-1514, XP002089524 cited in the application see the whole document ----- | 1-53 |

INTERNATIONAL SEARCH REPORT

International application No.

PCT/CA 98/00840

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claim(s) 45-49
is(are) directed to a method of treatment of the human/animal
body, the search has been carried out and based on the alleged
effects of the compound/composition.
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such
an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all
searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment
of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report
covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is
restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

information on patent family members

International Application No

PCT/CA 98/00840

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|-------------------------------------------|---------------------|----------------------------|---------------------|
| WO 9640945 A | 19-12-1996 | AU 695527 B | 13-08-1998 |
| | | AU 6117696 A | 30-12-1996 |
| | | CA 2223610 A | 19-12-1996 |
| | | EP 0832253 A | 01-04-1998 |
| | | US 5843913 A | 01-12-1998 |
| US 5614372 A | 25-03-1997 | EP 0811164 A | 10-12-1997 |
| | | WO 9626442 A | 29-08-1996 |
| WO 9617072 A | 06-06-1996 | AU 4594996 A | 19-06-1996 |
| | | EP 0797679 A | 01-10-1997 |
| | | US 5814482 A | 29-09-1998 |
| | | US 5843723 A | 01-12-1998 |
| | | US 5789245 A | 04-08-1998 |